

Rapid Communication

Neurofibrillary Tangles in Some Cases of Dementia Pugilistica Share Antigens with Amyloid β -Protein of Alzheimer's Disease

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Formalin-fixed, paraffin-embedded temporal lobe sections from eight former boxers' brains were examined using an immunohistochemical method with antibodies to amyloid β protein. In accord with recent observations in Alzheimer's disease, significant numbers of β -protein immunoreactive neurofibrillary tangles (NFT) were observed in three cases. Most of these immunoreactive NFTs appeared to be tombstone tangles, although not all such tangles were stained. This immunoreaction was completely abolished by preincubation of antibodies with synthetic β -protein peptides, and the identity of the immunostained NFTs was confirmed by polarization microscopy of sections counterstained with Congo red. However, it is not yet clear if the β -protein antigens are, in fact, an integral part of paired helical filaments. These observations, together with our recent finding of β -immunoreactive plaque-like lesions in dementia pugilistica, also emphasize the many similarities in pathology between this condition and Alzheimer's disease. (Am J Pathol 1990, 136:255–260)

Repeated head trauma experienced by boxers can lead to dementia pugilistica (punch-drunk syndrome) with a typical neuropathologic picture of degeneration of the substantia nigra, cerebellar scarring, abnormalities of the septum pellucidum, damage to the cerebrovascular system, and the diffuse presence of Alzheimer's neurofibril-

lary tangles (NFT) throughout the cerebral cortical grey matter (especially medial temporal lobe) and the brain stem.^{1–3} NFTs in dementia pugilistica are morphologically indistinguishable from those in Alzheimer's disease; both are composed of paired helical filaments (PHF),^{4,5} and antibodies to PHF-enriched fractions from patients with Alzheimer's disease react with NFTs in immunohistochemical studies of the brain in dementia pugilistica.⁶

In Alzheimer's disease, both senile plaque and cerebrovascular amyloid are composed of a 4.2-kd protein termed β protein (or A4),^{7,8} which is thought to be derived by proteolysis of a larger transmembrane glycoprotein precursor encoded on chromosome 21.⁹ Although earlier reports on dementia pugilistica stressed the relative absence of senile plaques,^{1,5} a recent study using a sensitive immunohistochemical method with formic acid pretreatment of brain sections has revealed many diffuse plaque-like lesions in this condition that were detected as areas of β -protein immunoreactivity with no histologic evidence of amyloid or of abnormal neurites.¹⁰ Similar lesions have recently been described in Alzheimer's disease,^{11–13} and they are also present in the absence of more mature senile plaques in young patients with Down's syndrome.^{14,15}

The chemical nature of the protein constituent(s) of PHFs in Alzheimer's disease has been controversial. Direct protein chemical analyses of isolated PHFs have identified β protein,¹⁶ tau,^{17,18} and ubiquitin¹⁹ as potential constituents, while immunohistochemical studies have shown that NFTs share epitopes with neurofilament proteins, microtubules, microtubule-associated protein

Supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, and grants for the Research of Dementia and for Primary Amyloidosis from the Ministry of Health and Welfare, Japan.

Accepted for publication November 14, 1989.

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Table 1. Selected Details of all Cases, Showing Those with β -Protein Immunoreactive NFTs in the Temporal Lobe

Case	Number	Age	Boxing career (yrs.)	Number of fights	Plaques*	Immunoreactive plaques†	NFT*	Immunoreactive NFT‡
1	48/72§	22	3	3 ⁺ (P)	—	—	—	—
2	88/72§	53	25	>100 (P)	—	++	+	—
3	120/71	58	5	>50 (A)	—	++	—	—
4	122/71	61	20	>100 (A)	+	++	+	—
5	124/71	62	20	400 (P)	—	++	+++	+
6	155/71	63	13	300 (P)	—	+++	+++	+
7	47/71	69	25	600 (P)	—	++	++	—
8	119/71	77	17	700 (P)	—	+++	+++	+

* Incidence of NFTs and senile plaques in cerebral cortex assessed by routine silver stains (von Braunmuhl's or Bielschowsky).

† Number of plaque-like lesions detected by β -protein immunohistochemistry of temporal lobe sections (from Roberts et al.¹⁰).

‡ Presence (+) or absence (—) of β -protein immunoreactive NFTs (present study).

§ Further details in Roberts et al.¹⁰ (cases 1 and 2 of these authors) or || Corsellis et al.¹

(A) amateur; (P) professional; (—) no lesions, (+) slight, (++) moderate, (+++) severe; + died during a fight.

MAP2, tau protein (for detailed references and discussion see review by Selkoe²⁰), and more recently ubiquitin.^{19,21–24} Most immunohistochemical studies, with or without formic acid pretreatment, have failed to find any immunoreactivity between antibodies to β protein and NFTs.^{11–15,25–28} However, recently it was reported that some extracellular tombstone tangles in brains of patients with Alzheimer's disease reacted with antibodies to a synthetic β -protein peptide.²⁹ Here we describe an apparently similar finding in the brains of three cases of dementia pugilistica.

Materials and Methods

Formalin-fixed tissue from the temporal lobe was obtained from six of the boxers' brains originally reported by Corsellis et al.,¹ and from two additional cases (Table 1). Each case had already undergone extensive neuropathologic examination at Runwell Hospital, U.K., and had been investigated to establish details of the boxer's career. Paraffin-embedded sections were immunostained for β protein using the avidin-biotin complex technique (Vector Labs, Burlingame, CA). Some sections from each case were pretreated for 10 minutes with 90% formic acid to enhance β -protein immunoreactivity.^{12,28} The primary antibodies were (1) monoclonal antibody 4D12/2/6 to residues 8–17 of β protein²⁶ (1:500 ascites fluid), and (2) 1:500 polyclonal rabbit antiserum to residues 1–24 of β protein.³⁰ Positive immunoreactivity was detected with 3,3'-diaminobenzidine tetrahydrochloride. The sections were counterstained with 1% aqueous Congo red followed by hematoxylin. To demonstrate immunospecificity of staining a 1:500 dilution of antibody was preincubated overnight at 5°C with 100 μ M of the appropriate synthetic peptide.

Results

No immunoreactive NFTs and only a few typical senile plaques (case 4 only) were observed without formic acid

pretreatment of the brain sections. After formic acid pretreatment, many areas of β -protein immunoreactive diffuse plaque-like lesions were revealed in seven of the eight former boxers' brains available for the present study, as described in detail elsewhere.¹⁰ In addition, three cases (Table 1) showed immunoreactive structures closely resembling NFTs that were detected using both antibodies to residues 8–17²⁶ and 1–24³⁰ of β protein (Figure 1). These immunoreactive structures were observed only in cases containing large numbers of NFTs demonstrable by routine silver impregnation methods (Table 1) and the majority of them were present in areas of temporal cortex containing very high concentrations of NFTs, as seen on adjacent silver-stained sections (Figure 1E) or on immunostained sections counterstained with Congo red. The impression that some NFTs were positively stained was confirmed by polarization microscopy of immunostained sections with Congo red counterstain. Often the typical Congo red birefringence of the NFTs could still be observed together with the peroxidase staining product, although the intensity of this birefringence was reduced (Figure 1D). Many of the stained structures were obviously fibrous in nature, with some showing the characteristic flame shape of Alzheimer's disease NFTs, but sometimes it was difficult to decide whether the stained structure was a tangle or merely an intensely immunoreactive neuronal perikaryon. This staining pattern was clearly different from the β -protein immunoreactive granular profiles described in many widely distributed neurons and other cell types by Stern et al.,³¹ or the reaction with lipofuscin granules described by Banerjee et al.³² Usually the diffuse plaque-like areas and immunostained NFTs were easily distinguished from each other (Figure 1A, B). On close inspection, the vast majority (but not all; see for example Figure 1A) of the immunoreactive NFTs appeared to be tombstone tangles or ghost tangles (ie, naked NFTs in which the surrounding neuron has completely degenerated) because they had no associated neuronal nucleus

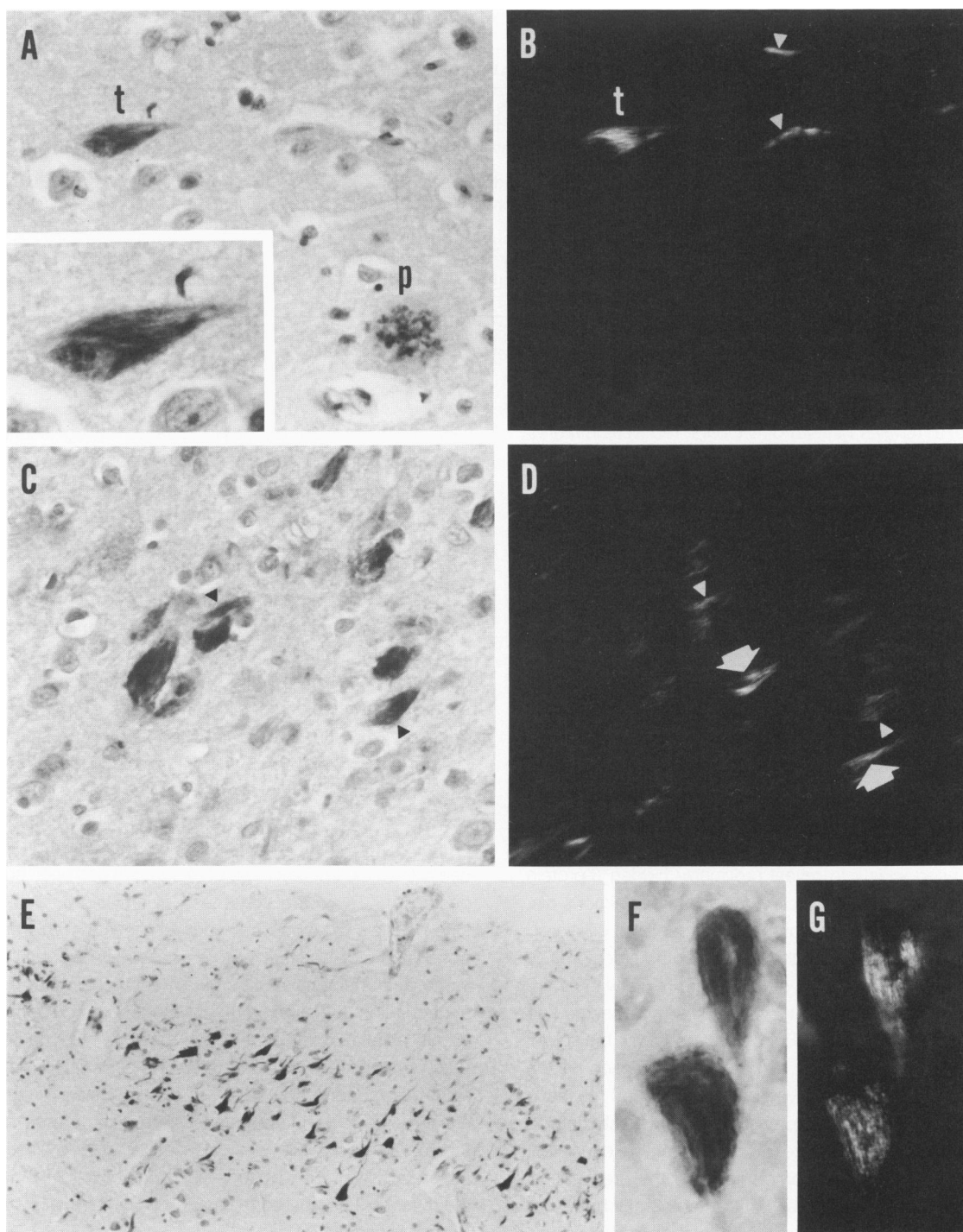


Figure 1. β -protein immunoreactivity of NFTs in dementia pugilistica, with Congo red counterstain. **A:** Immunoreactive tangle (t) from case 8 is easily distinguished from a diffuse plaque-like lesion (p) ($\times 320$). Inset at higher magnification shows the fibrous nature of the tangle (4D12/2/6 antibody) ($\times 640$). **B:** Polarized light view of (A) reveals Congo red birefringence of the immunoreactive tangle (t) and some additional nonreactive NFTs (arrowheads). **C:** and **D:** are normal and polarized light views of case 8, with examples of reactive tangles indicated by arrowheads, and nonreactive tangles indicated by arrows (β 1-24 antibody) ($\times 320$). **E:** Methenamine silver stain⁴⁰ shows a high concentration of NFTs in case 8, some of which proved to be immunoreactive on adjacent immunostained sections ($\times 160$). **F:** and **G:** are normal and polarized light views of two immunoreactive NFTs from case 6 (4D12/2/6 antibody) ($\times 640$).

or any evidence of a surrounding membrane. Not all extracellular tangles were stained, however, with the exact proportion varying from case to case and from region to re-

gion within the same brain section. In some medium-power fields of temporal cortex from case 8 (which had the greatest number of immunoreactive NFTs) 40% to

Table 2. *Further Neuropathologic Details of the Three Cases Showing β -Protein Immunoreactive NFT*

Case	Number	Brain weight	Cavum width	Changes in the substantia nigra	Distribution of NFTs
5	124/71	1090 g	4 mm	Slight loss of pigmented cells with moderate NFT formation	Many in uncus, amygdaloid nucleus, hippocampus, parahippocampal gyrus, and sporadically elsewhere
6	155/71	1260 g	5 mm	Severely degenerated with numerous NFTs present	Many throughout frontal and temporal cortex, particularly anteriomedial temporal grey matter
8	119/71	960 g	7 mm	Many pigmented cells lost with numerous NFTs present	Many throughout whole cortex, particularly medial temporal grey matter

Summarized from Corsellis et al.¹ All cases had a septal cavum (width shown) with fenestrated leaves, and cases 6 and 8 showed tonsillar scarring of the cerebellum. All cases showed progressive dementia, and Parkinsonian symptoms were noted in case 6.

50% of all tangles visualized by Congo red were immunostained, but in other regions from the same tissue block this proportion was much lower. Control sections tested with antibodies blocked by preincubation with their cognate synthetic peptide showed no immunoreactivity.

Discussion

We found that a well-characterized monoclonal antibody to residues 8–17 of β protein²⁶ and a polyclonal antibody to residues 1–24³⁰ both reacted with some NFTs in an immunohistochemical study of cerebral temporal cortex from three of eight former boxers' brains. NFTs showed such immunoreactivity only after pretreatment of the brain sections with formic acid, a method that recently has been used widely to reveal previously hidden β -protein epitopes in diffuse plaque-like lesions in Alzheimer's disease,^{11–13} Down's syndrome,^{14,15} dementia pugilistica,¹⁰ and in fewer numbers in the nondemented elderly.³³ The immunospecificity of this reaction with NFTs was established by the complete inhibition of staining observed after preincubation of both kinds of antibody with the appropriate synthetic peptide. A detailed description of the three positive cases is given by Corsellis et al¹ (cases 2, 3, and 7 of these authors); all of them showed clinical and neuropathologic evidence of brain damage consistent with punch-drunk syndrome, including the presence of numerous NFTs, particularly in the temporal lobe (Table 2). Fewer NFTs, which were all nonreactive to β -protein antibodies, were present in three cases, while NFTs could not be detected at all in two cases that served as negative controls (Table 1). These results, together with the finding that β -protein epitopes were mainly associated with extracellular tombstone tangles, suggest that β -protein immunoreactivity of NFTs is restricted to cases with particularly large numbers of these lesions, and in cases in which a significant proportion of lesions have consequently reached the final extracellular stage in their evolution.

A most important question concerns the significance of these observations in relation to the chemical composition of PHF. There have been no direct protein chemical analyses of PHFs isolated from boxers' brains, but the available ultrastructural and immunohistochemical evidence does suggest that PHF in this condition are identical with those in Alzheimer's disease.^{5,6} The finding that some extraneuronal tombstone tangles in dementia pugilistica are antigenically related to β protein parallels recent observations in some limited cases of Alzheimer's disease²⁹ (and written personal communication from M. Landon, July 24, 1989) and provides further evidence for the similarity of PHFs in these two conditions. On the basis of amino acid compositional^{34,35} and N-terminal amino acid sequence data¹⁶ obtained from PHF-enriched fractions prepared from Alzheimer's disease brains, and more recently from Guam-Parkinson dementia,³⁶ it has been suggested that PHFs are composed of amyloid β protein. One possible explanation for our results is that this proposal is correct, and that β -protein epitopes are obscured in most intraneuronal and some extraneuronal NFTs due to decoration of the paired helical shafts with fuzzy amorphous material containing neurofilament, MAP2, tau, or ubiquitin epitopes; only when this PHF-associated material is degraded by the action of extracellular proteases are the β -protein epitopes accessible in the tombstone tangles. This possibility was discussed in detail by one of us previously.³⁷ The limited immunoreactivity of tombstone tangles compared to intracellular NFTs would support this notion.³⁷

The amino acid sequence data suggesting that PHFs consist of β protein have been questioned due to the likely contamination of PHF preparations with senile plaque or cerebrovascular amyloid fibrils.^{20,27} Other protein chemical analyses of PHFs have indicated that these structures contain tau^{17,18} or ubiquitin¹⁹ components. Thus an alternative interpretation of our results is that PHFs are composed of tau (this seems a more likely possibility than ubiquitin), or another as-yet unknown protein, and that mate-

rial containing β -protein epitopes is deposited onto the surface of some of the tombstone tangles after they are exposed to the extracellular environment. This situation would be similar to glial fibrillary acidic protein, which can be detected by immunohistochemical methods in extracellular but not intracellular tangles due to penetration of NFTs by astroglial processes only after they are released into the extracellular space.^{38,39}

Which of these two alternatives is correct? Further careful protein chemical analyses of highly purified PHFs, which have been stripped of any associated non-PHF material, and have been quantitatively solubilized, with the accurate calculation of yields, will be required to reach a definitive conclusion on the chemical nature of PHFs. Immunogold studies of tombstone tangles using antibodies to β protein will provide some information on the precise ultrastructural nature of the tissue component(s) in NFTs containing β -protein epitopes. Only if the gold particles are precisely located on the shafts of the PHFs will this provide strong support for an integral β -protein component.

Finally, these results, together with the finding of β -amyloid plaque lesions in dementia pugilistica,¹⁰ emphasize the many similarities in pathology between dementia pugilistica, Alzheimer's disease, and other related disorders (eg, Down's syndrome) with β -amyloid deposits in the brain.

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Acknowledgment

The authors thank Mr. Kato for his expert assistance with the photography.